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pH Dependent Acetate Injury in *Staphylococcus aureus*: Role of Temperature

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Injury of Staphylococcus aureus 196E exposed to acetate buffer (pH 4.4–4.6) was temperature dependent, with injury occurring at 40°C, death at 45°C, and little or no effect at ≦35°C. Injury was also dependent on acetate concentration and pH; equivalent phosphate buffers did not produce an injury response. Cells pre-exposed to acid acetate buffer at 20°C and then placed in pH 7.0 acetate buffer at 40°C were injured, but no injury occurred in cells not previously exposed to acidic conditions when placed in neutral buffer at 40°C. Acetate (0.2 M) blocked glucose utilization in 20°C and 40°C incubated cells at pH 4.4, but not at pH 6.1. Glucose was metabolized by S. aureus in phosphate buffer at both pH 4.5 and 6.0.

Introduction

Few investigators have reported on acid injury in bacteria. ROTH and KEENAN (12), using various organic or mineral acids added to broth to produce ranges of pH from 3.0 to 4.3, and PRZYBYLSKI and WITTER (11), using acetate buffer at pH 4.2 (0.3 M), demonstrated that acid-injured strains of *Escherichia coli* were unable to grow on violet red bile agar but were able to grow on nutrient agar or trypticase soy agar (on which injured cells can repair). PRZYBYLSKI and WITTER (11) also found that repair was brought about by incubation of the injured cells in potassium phosphate buffer (0.04 M, pH 8.0). Increasing the molarity of the buffer, decreasing the pH, or substituting sodium for potassium in the buffer caused a decrease in the number of repaired cells. BLANKENSHIP (2) found that *Salmonella bareilly* was injured (failed to grow on violet red bile agar) when suspended in 0.2 M acetate buffer at pH 3.1. Unlike acid acetate injured *E. coli,* the injured salmonellae required a medium containing amino acids to effect repair.

SMITH and PALUMBO (13) showed that *Staphylococcus aureus* present in fermenting sausages would undergo injury as demonstrated by failure of the injured cells to grow on agar medium containing 7.5% NaCl. The injury was assumed to be due to lactic acid produced by the starter culture used in the fermentation. The acid injury was shown also to be temperature dependent with no further injury taking place when the fermented sausages were transferred to 5°C (13). SMITH *et al.* (14) studied injury to *S. aureus* suspended in 0.2 M acetate buffer at pH 4.6 and showed that sugars or polyols that lower water activity produced a substantial decrease in injury. In the present study, the influence of temperature on injury in acid acetate buffer was determined in *S. aureus* 196E.

Materials and Methods

Preparation of cells

Staphylococcus aureus 196E was grown in 100 ml tryptic soy broth (Difco*) incubated on a rotary shaker (200 rpm) for 16 h at 37°C. Cells were harvested by centrifugation (16,000 × g at 0°C) and washed with three successive portions of sterile potassium phosphate buffer (0.1 M, pH 7.2). The cells were then suspended in 3-ml sterile distilled water.

Injury experiments

Acetic acid (1.0 M) and sodium acetate .3 H_2O (1.0 M) solutions were mixed and diluted to give buffers of appropriate pH and molarity (5). $NaH_2PO_4 \cdot H_2O$ (138 g) was dissolved in 500 ml distilled water, adjusted to pH 4.5 with 1 N NaOH, and made up to 1-liter volume to give a 1 M solution. It was then diluted with distilled water to give the appropriate molarity. Experimental flasks containing 50 ml buffer were placed in a constant temperature water bath to maintain the flask contents at the desired temperature. The temperature of the flasks was monitored by insertion of a thermocouple beneath the liquid surface; flask contents were agitated using a magnetic stirrer. When the flask contents were equilibrated to the appropriate temperature, washed cells were added to give an initial count of approximately 5×10^8 cells/ml. Approximately 3 min were required for temperature equilibration after addition of the cells.

Determination of injured cells

At intervals, 0.1-ml of cell suspension was removed from each injury flask and transferred to 9.9-ml sterile peptone (0.1%; Difco) water blanks. Appropriate dilutions were surface plated onto tryptic soy agar (TSA; Difco) plus 1% sodium pyruvate (TSAP) and TSA plus 7% NaCl (TSAS)

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utilizing a spiral plater (Spiral Systems Marketing, Bethesda, MD). Both injured and noninjured cells grow on TSAP whereas only noninjured cells grow on TSAS; injured *S. aureus* are no longer salt-tolerant (15). The plates were counted after 48 h at 37°C.

Determination of pH and glucose

The pH of the experimental flasks was determined utilizing an Orion Research pH meter (Model 601A) with a combination glass electrode (Orion 91-04). Glucose was determined by using the cT-V™ direct glucose reagent kit (Stanbio Lab., Inc., San Antonio, TX).

Respirometric studies

Oxygen uptake by *S. aureus* utilizing glucose as the substrate was investigated using 15 ml single-side arm Warburg flasks attached to a Gilson differential respirometer. Each flask contained a total volume of 3 ml: sodium phosphate buffer (0.2 M, pH 4.5 or 6.0) or sodium acetate buffer (0.2 M, pH 4.4 or 6.1) and 0.01 mM glucose were placed in the main compartment; 0.2 ml of 40% KOH in the center well; and washed cells of *S. aureus* (approximately 6-mg dry weight) were placed in the side arm. Flasks were equilibrated at 40°C, cells were tipped in and uptake of O_2 determined. Endogenous values were substracted.

Results

Under acidic conditions, injury and lethality in *S. aureus* is affected strongly by temperature. After 1-h treatment in 0.2 M acetate buffer at pH 4.5, *S. aureus* was not injured at temperatures ranging from 5° to 35°C **(Tab.1).** If the Δ count was ≧1.0, then the system was deemed injurious. At 40°C, only injury was observed, however, at 45° and 50°C, both cell injury and death (death is shown by a decrease in counts on TSAP; the zero time population was 3 to 5×10^8/ml) were observed **(Tab.1).** When the pH of the acetate buffer was 6.8, counts on TSAP and TSAS were similar over a range of 20° to 50°C, indicating that neither injury nor cell death had occurred **(Tab.1).**

Tab.1 Influence of temperature on injury to *S. aureus* 196E in acetate buffer

Temperature, °C	Log_{10} count after 1 h treatment; plated on		
	TSAP	TSAS	Δ Count
0.2 M acetate buffer, pH 4.5			
5	8.22	8.32	–0.10
9	8.18	8.18	0.00
14	8.49	8.91	–0.50
25	8.37	8.53	–0.16
30	8.35	8.22	0.13
35	8.57	8.11	0.46
40	8.32	6.46	1.76
45	6.51	3.86	2.65
50	3.44	1.44	2.00
0.2 M acetate buffer, pH 6.8			
20	8.56	8.64	–0.08
30	8.49	8.57	–0.08
40	9.04	8.68	0.36
50	8.46	8.39	0.07

In addition to temperature, the concentration of acetate buffer was important in producing injury. At pH 4.4 and 40°C, there was increasing injury and killing as the molarity of the buffer increased **(Tab.2).** However, at pH 6.1, an increase in acetate concentration had little effect. That acetate is the

Tab.2 Influence of acetate or phosphate concentration on injury to *S. aureus* 196E after 1 h at 40°C

Molarity	Log_{10} count; plated on		
	TSAP	TSAS	Δ Count
pH 4.4, acetate buffer			
0.05	9.03	8.92	0.11
0.1	8.95	8.58	0.37
0.2	8.84	7.15	1.69
0.3	8.56	6.39	2.17
0.4	9.02	4.72	4.30
0.5	8.34	3.97	4.37
0.6	7.52	4.16	3.36
0.7	7.07	3.25	3.82
pH 6.1, acetate buffer			
0.1	8.88	8.92	–0.04
0.2	9.23	9.11	0.12
0.4	9.11	9.04	0.07
0.6	8.87	8.83	0.04
pH 4.5, phosphate buffer			
0.2	8.88	8.88	0.00
0.4	9.06	8.97	0.09
0.6	8.64	8.54	0.10
0.8	8.90	8.79	0.11

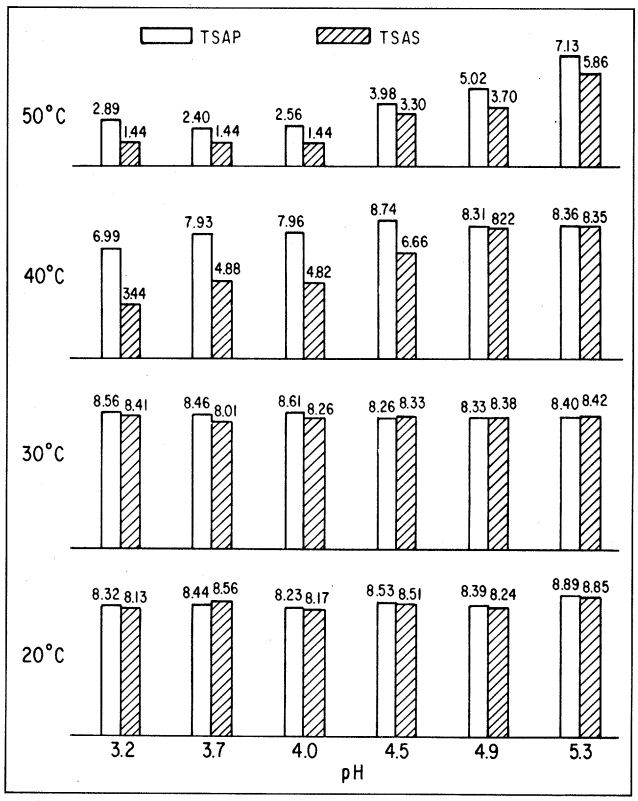

Fig. 1 Effect of temperature and pH on injury to S. aureus 196E in 0.2 M acetate buffer.

Numbers in each bar represent log_{10} *S. aureus*/ml

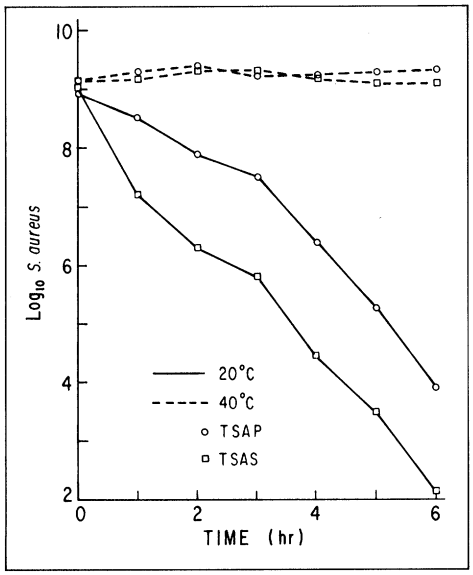

Fig. 2 Influence of temperature on acid injury in S. aureus 196E as a function of time (0.2 M acetate buffer, pH 4.4).

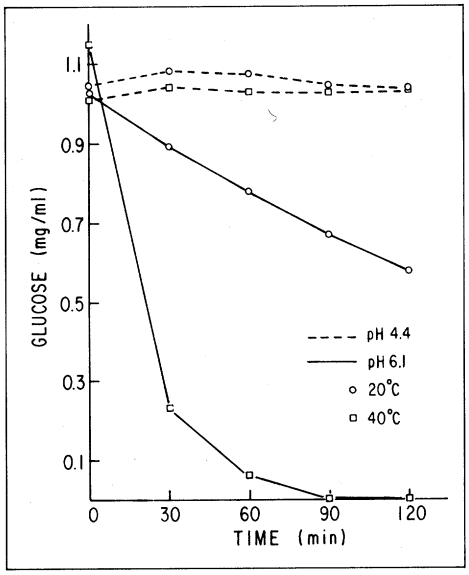

Fig. 4 Influence of temperature and pH on utilization of glucose by S. aureus 196E in 0.2 M acetate buffer.
The experimental procedure was similar to the injury procedure except that 0.1% glucose was present in the acetate buffer.

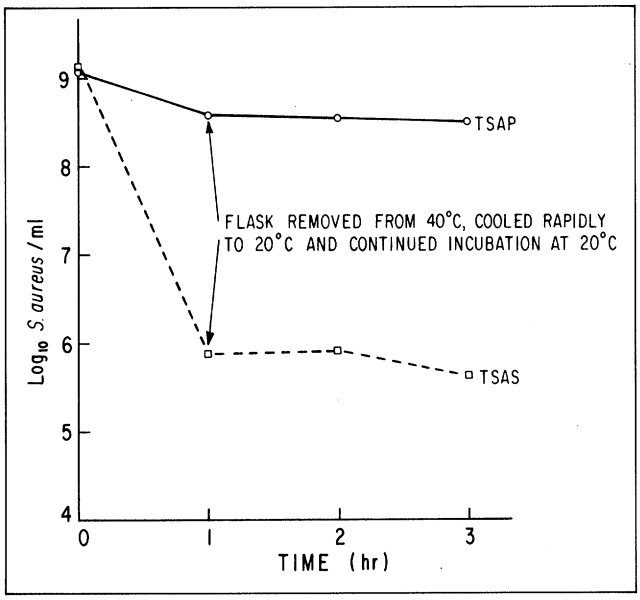

Fig. 3 Effect of incubation at 20°C on S. aureus 196E previously injured at 40°C (0.2 M acetate buffer, pH 4.6).

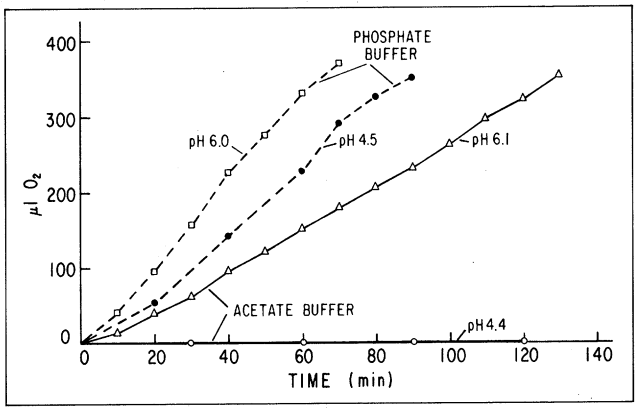

Fig. 5 Influence of buffer composition and pH on O_2 uptake with glucose by S. aureus 196E (40°C).

toxic moiety and not pH, *per se,* is demonstrated by comparing phosphate and acetate. Under acidic conditions (pH 4.5), phosphate did not lead to injury in *S. aureus* regardless of the molarity whereas acetate was increasingly injurious as the concentration was increased at low pH **(Tab.2).**

At 20° and 30°C, 0.2 M acetate buffer over a pH range of 3.2 to 5.3, caused neither death nor injury **(Fig.1).** At 40°C, death of the cells was observed at pH 3.2 (approximately 1.5 logs) but was minimal at other pH values tested. Injury was observed at 40°C at pH values ranging from 3.2 to 4.5, but neither injury nor death was found at pH 4.9 or 5.3. However, incubation of *S. aureus* under acidic conditions at 50°C led to injury and/or death regardless of pH **(Fig.1).**

Even after 6-h incubation at 20°C in 0.2 M acetate buffer at pH 4.4, cells were not injured **(Fig.2).** Prolonged incubation at 40°C caused both death and injury. Data presented in **Fig.3** indicate that cells injured at 40°C under acidic conditions do not undergo further injury (that is, there was no further decrease in TSAS counts), upon a sudden shift to 20°C.

Prior exposure of *S. aureus* to pH 4.6 acetate buffer for 5 h at 20°C resulted in injury when those cells were resuspended in pH 6.9 acetate buffer and held for 1 h at 40°C **(Tab.3).** Pretreatment of the cells at 5°C for 5 h did not lead to injured cells when exposed to neutral buffer at 40°C. Pretreatment at 20°C for 2.5 rather than 5 h did not give injured cells **(Tab.3).** However, pretreatment of *S. aureus* in acid buffer at 20°C for 5 h predisposed the cells to injury when those cells were placed in neutral buffer at 40°C **(Tab.3)** even though exposure of normal (not pretreated) cells to neutral buffer at 40°C did not lead to injury **(Tab.1).**

Decreasing the growth temperature of *S. aureus* has been reported to induce changes in the composition of membrane lipids (9). To determine if a lowered growth temperature

Tab.3 Effect of pretreatment of *S. aureus* 196E at 5° or 20°C in acid buffer on injury when transferred to neutral buffer at 40°C

Treatment of cells	pH	Temperature, °C	Time, h	Log_{10} increase in number of injured cells
1. 0.2 M acetate buffer[a]	4.6	5	5	0.0
2. 0.2 M acetate buffer[a]	4.6	20	2.5	0.0
3. 0.2 M acetate buffer[a]	4.6	20	5	0.0
4. 0.2 M acetate buffer plus cells from #1[b]	6.9	40	1	0.16
5. 0.2 M acetate buffer plus cells from #2[b]	6.9	40	1	0.09
6. 0.2 M acetate buffer plus cells from #3[b]	6.9	40	1	2.08
7. 0.2 M acetate buffer plus untreated cells	4.6	40	1	2.43

[a]Cells were pretreated in 0.2 M acetate buffer, pH 4.6 for 2.5 or 5 h at either 5° or 20°C.
[b]Cells were centrifuged free of pretreatment buffer and resuspended in 0.2 M acetate buffer, pH 6.9 held at 40°C.

Tab.4. Effect of growth temperature of *S. aureus* 196E on acid injury at 20° or 40°C

Cell growth Temperature, °C	Injury temperature, °C	Log_{10} increase in number of injured cells at 60 min
20	20	0.0
37	20	0.06
20	40	1.74
37	40	2.50

with its ensuing change in membrane lipids would influence the extent of acid injury, *S. aureus* was grown both at 20°C and at 37°C and subjected to acid injury conditions. Data in **Tab.4** indicate that neither 20° nor 37°C grown cells were injured at pH 4.6 at 20°C but both types of cells were injured when the temperature of injury treatment was 40°C.
In acetate buffer containing 0.1% glucose, *S. aureus* was able to utilize the sugar, regardless of temperature (20° or 40°C), when the buffer was poised at pH 6.1 **(Fig.4);** at pH 4.4, glucose was not metabolized. Similarly, with glucose as the substrate, O_2 uptake was demonstrated in acetate buffer at pH 6.1 but not at pH 4.4 **(Fig.5).** However, with phosphate buffer, O_2 uptake was demonstrated at both pH 6.0 and 4.5.

Discussion

The present study demonstrated that pH dependent acetate injury in *S. aureus* is temperature dependent. The data indicated that under acidic conditions, acetate injury did not occur at temperatures ranging from 5° to 35°C. Injury was observed in *S. aureus* when the cells were exposed to acetate under acidic conditions at 40°C, however. At temperatures of 45°C and above, cell death (as demonstrated by decreased TSAP counts) appeared to be the major phenomenon rather than injury. Even when the time of exposure of *S. aureus* to acid acetate buffer was extended from 1 h to 6 h at 20°C, cellular injury was not observed. When the temperature was increased to 40°C, injury followed by death was observed during the 6-h period. Thus, a strong relationship between *S. aureus* injury (and death) in acid acetate buffer and the temperature of exposure is indicated.
In addition to temperature, the molarity of the acetate buffer was an important determinant of injury (and death) in *S. aureus*. At pH 4.4 and 40°C, increasing concentrations of acetate led to increasing injury and death. However, increasing the molarity of acetate buffer at pH 6.1 had little or no effect on the cells. Regardless of the molarity of phosphate buffer at pH 4.5 (40°C), injury was not found, thereby indicating that the injury noted here is a pH dependent acetate effect. Other workers have noted that increasing the molarity of acetate increased deleterious effects on cells. PRZYBYLSKI and WITTER (11) found that injury and death increased in *E. coli* as the acetate buffer concentration was increased from 0.1 to 1.0 M at pH 4.6. The $D_{40°C}$ value at pH 4.7 for *S. typhimurium* decreased from 9.17 when the acetate buffer was 0.1 M to 2.97 when the buffer was 0.4 M (10).
Expression of injury immediately ceased when the temperature was abruptly changed from 40° to 20°C, and incubation at the lower temperature for an additional 2-h period did not lead to further injury. SMITH and PALUMBO (13) noted cessation of injury in fermented sausages when the sausages were transferred from the 35°C fermentation chamber to 5°C storage. The injury to *S. aureus* ceased even though the acid concentration in the sausages was high. Reincubation of the low temperature stored sausages at 35°C led to the resumption of the injury process and, ultimately death of the staphylococci (13). Also, BLANKENSHIP (2) noted that little or no injury took place at temperatures below 10°C in *S. bareilly* exposed to acidic acetate buffer. The basis for the lack of expressed acid injury in *S. aureus* at low temperatures is not readily apparent but several hypotheses may be formulated to explain such phenomena.

(a) *The expression of acid acetate injury is dependent on cells actively metabolizing at 40°C.* Respirometric studies with washed cells of *S. aureus* indicated that no uptake of O_2 occurred in the presence of glucose in 0.2 M acetate buffer at pH 4.5, nor was there disappearance of glucose regardless of the temperature (20° or 40°C). However, in acetate buffer at 6.1, glucose was utilized at both temperatures. Thus, the metabolism of glucose does not appear to be required for the expression of acid acetate injury at 40°C.
An alternate possibility is that there is a temperature-dependent acetate-induced production of a toxic component. BLUHM and ORDAL (3) reported that butanediol dehydrogenase in *S. aureus* is readily inactivated during sublethal heat injury, leading to the accumulation of diacetyl and acetoin. BUCHANAN and SOLBERG (4) demonstrated that exogenously supplied acetate stimulated acetoin production in *S. aureus*. It is possible that this effect is temperature dependent, and at the higher temperature, there is an accumulation of diacetyl and acetoin due to the inactivation of butanediol dehydrogenase. Diacetyl has been shown to be toxic to *S. aureus* (8).

(b) *Less un-ionized acetic acid is available to penetrate the bacterial cells at lower temperatures as compared to 40°C.* The undissociated acetic acid molecule is inhibitory to the growth of microorganisms (7), and presumedly, the same

chemical species is responsible for injury. The pKa of acetic acid is 4.77 at 5°C, 4.76 at 10° to 35°C, and 4.77 at 40°C (16). The amount of un-ionized acetic acid available to produce injury at a particular pH will be essentially the same over a temperature range of 5° to 40°C.

(c) *The physicochemical nature of the bacterial membrane is different at 20°C as compared to 40°C and less acetic acid can penetrate the cell to cause injury.* Phospholipids in *Vibrio parahaemolyticus* show a saturated/unsaturated fatty acid ratio of 0.51 with 21°C grown cells and 0.91 with 37°C grown cells. The increase in saturated fatty acid content associated with growth at 37°C is correlated with an increase in heat resistance of *V. parahaemolyticus* (1). Similarly, an increase in saturated phospholipids with increasing growth temperature in *E. coli* was reported by HAEST *et al.* (6). The *E. coli* membrane shows decreased permeability to solutes when the saturated phospholipid content was increased. Interestingly, changes in the saturated/unsaturated fatty acid ratios of membrane lipids did not occur in *S. aureus* grown at high or low temperatures (9); however, there was an increase in the polar carotenoid content when staphylococci were grown at low temperatures. JOYCE *et al.* (9) suggested that the increased polar carotenoid content in *S. aureus* grown at low temperatures serves a similar role as does increased levels of unsaturated phospholipids in other bacteria by allowing membrane permeability to function at low temperatures. The nature of the membrane of *S. aureus* grown at 37°C may allow acetic acid to penetrate cells suspended in acetate buffer at 40°C but not at 20°C. Growing *S. aureus* at 20°C, therefore, should alter the membrane such that increased permeability of acetic acid into cells suspended at 20°C may occur. The data presented in **Tab. 4** indicate, however, that *S. aureus* grown at 20°C is no more susceptible to acid acetate injury at 20°C than are 37°C grown cells and both 20° and 37°C grown cells are injured at 40°C.

Data presented in **Tab. 3** indicate that acid does penetrate the staphylococcal cell membrane at 20°C even though injury is not expressed. Exposure of *S. aureus* to acidic acetate at 20°C for 5 h (during which time no injury is detected) leads to expression of injury when the cells are washed free of acidic buffer and placed in neutral buffer at 40°C. Penetration of acid into the cells at 20°C and pH 4.6 predisposes them in some manner so that injury is expressed when the temperature and pH are shifted upwards (40°C and pH 7).

(d) *Denaturation of proteins or other cellular macromolecules of S. aureus is limited at low temperatures over a range of 3.2 to 5.3.* Acid injury in *S. bareilly* was not detected at 10°C or lower. BLANKENSHIP (2) showed that *S. bareilly,* acid injured at 30°C, contained decreased levels of extractable protein because the cellular proteins were apparently rendered insoluble by the acid injury conditions at 30°C. Activities of lactic dehydrogenase and glucose-6-phosphate dehydrogenase were also decreased. BLANKENSHIP (2) suggested that protein denaturation by the acid treatment was an important part of acid injury; the denaturation was reversible because repair of cellular damage could occur under proper conditions.

It is possible that little or no denaturation of macromolecules essential to the survival of *S. aureus* occurs at low temperatures in acetate buffer at low pH. As the temperature is increased to 40°C, injury takes place due to reversible denaturation of some essential cellular component(s); the denaturation is reversible because injured cells repair and form colonies on TSAP. At higher temperatures (≧50°C), death of the cells result due to irreversible denaturation of macromolecules under acidic conditions.

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